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<p>(21) International Application Number: PCT/GB97/02948 (22) International Filing Date: 5 November 1997 (05.11.97) (30) Priority Data: 9623149.3 7 November 1996 (07.11.96) GB (71) Applicant (for all designated States except US): THE VICTORIA UNIVERSITY OF MANCHESTER [GB/GB]; Oxford Road, Manchester M13 9PL (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): VADGAMA, Pankaj, Maganlal [GB/GB]; 4 The Sidings, Drywood Avenue, Worsley, Manchester M28 4QA (GB). MAINES, Andrew, David [GB/GB]; 61 Whitelake Avenue, Flixton, Manchester M41 5GN (GB). (74) Agent: ATKINSON, Peter, Birch; Marks &amp; Clerk, Sussex House, 83-85 Mosley Street, Manchester M2 3LG (GB).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>	
<p>(54) Title: BIOSENSOR INCORPORATING A SURFACTANT</p>		
<p>(57) Abstract</p> <p>A biosensor for the determination of a selected analyte in a sample comprises an enzyme layer incorporating an enzyme which is capable of interacting with said analyte to provide a detectable change, detecting means on one side of said layer for detecting said change, and an outer diffusion limiting barrier membrane which is provided on the opposite side of said layer to the detecting means. The membrane incorporates a surfactant so as to render it permeable to said analyte and the enzyme layer incorporates at least one substance essential to enzyme activity (e.g. a co-factor). The nature and amount of the surfactant are such that the membrane inhibits release of the substance essential to enzyme activity into the sample whilst retaining permeability to the analyte.</p>		

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**BIOSENSOR INCORPORATING A SURFACTANT**

The present invention relates to a sensor and more particularly to a biosensor.

Biosensors are used for determining the presence and/or amount of a selected analyte in a sample. The particular type of biosensor to which the present invention relates comprises an enzyme which is specific for the analyte to be determined and which interacts therewith to produce a chemical change indicative of the presence and/or amount of the analyte. The change may be detected by any suitable means, e.g. an electrode arrangement. Thus, for example, an enzyme electrode system for the determination of glucose comprises an enzyme layer incorporating glucose oxidase which catalyses the oxidation of glucose by molecular oxygen to produce gluconic acid and hydrogen peroxide, either of which may be determined by an amperometric electrode.

In use of such biosensor, it is necessary that the enzyme be provided with any substance essential to its activity for effecting the chemical change which forms the basis of the detection procedure. Examples of such substances include enzyme co-factors and electron transfer mediators. Such substances are generally provided in the sample being analysed and, in the case where the biosensor includes a diffusion limiting membrane between the enzyme and sample, diffuse through the membrane so as to be available for the enzyme.

The need to provide such substances in the sample is however a considerable disadvantage for several reasons. Firstly, the need to add the substances to the analyte is an extra step in the detection procedure. Secondly, it is necessary to use substantially more of the substances than actually required by the enzyme. This is particularly the case if measurements on the sample are being made under continuous flow conditions.

It is therefore an object of a first aspect of the present invention to obviate or mitigate the abovementioned disadvantages.

According to a first aspect of the present invention there is provided a biosensor for the determination of a selected analyte in a sample, the bio-sensor comprising an enzyme layer incorporating an enzyme which is capable of interacting with said analyte to provide a detectable change, detecting means on one side of said layer for detecting said change, and an outer diffusion limiting barrier membrane which is provided on the opposite side of said layer to the detecting means, said membrane incorporating a surfactant so as to render it permeable to said analyte wherein said enzyme layer incorporates at least one substance essential to enzyme activity and the nature and amount of said surfactant is such that the membrane inhibits release of said substance into the sample whilst retaining permeability to the analyte.

We have found, and this forms the basis of the present invention, that surfactant incorporating membranes may be produced which are capable of permitting sufficient diffusion of analyte species whilst nevertheless inhibiting diffusion of substances essential to enzyme activity (e.g. enzyme co-factors, electron transfer mediators, coenzymes and activators). Using such membranes it is possible to produce biosensors in which substances required for enzyme activity are provided in the enzyme layer rather than in the sample medium containing the analyte.

Non-limiting examples of enzymes which may be used in the sensor of the invention are given in Table 1 below together with examples of co-factors etc. essential for their activity which are incorporated, together with the enzyme in the enzyme layer.

**TABLE 1**

<b>ENZYME</b>	<b>CO-FACTORS ETC.</b>
(i) Pyruvate Oxidase	Cocarbonylase (thiamine pyrophosphate chloride), $Mg^{2+}$ , flavin adenine dinucleotide (FAD)
(ii) Lactate Dehydrogenase	Diaphorase, potassium ferricyanide, $NAD^+$
(iii) Malate Dehydrogenase	Diaphorase, potassium ferricyanide, $NAD^+$

The combinations (i), (ii) and (iii) may be used in sensors for determination of pyruvate, lactate and malate respectively.

Other cofactors may be additionally or alternatively used within the enzymes described in Table 1. These include:-

for (ii) NADH

for (iii) NADH, NADPH and/or  $NADP^+$

Many other enzymes requiring cofactors/mediators may be suitably used according to the invention. Further examples include a variety of other dehydrogenase enzymes (e.g. glucose, alcohol, fructose or glutamate dehydrogenases) which require cofactors similar to (ii) or (iii) in Table 1 above. Kinases (such as pyruvate kinase or protein kinase) may also be used. Such kinases usually require cofactors such as  $Mg^{2+}$  ADP and/or ATP. In fact, membranes incorporating a surfactant according to the invention greatly expand the range of enzymes that may be used in such sensors.

The surfactant may be a non-ionic, cationic, anionic or zwitter ionic surfactant and will generally be present in the membrane in an amount of 10% to 60% based on the total weight of the membrane.

A preferred cationic surfactant is a methyl mixed trialkyl quaternary ammonium salt (e.g. the chloride) in which the said alkyl groups each have up to 100, preferably 10 to 100 carbon atoms. One example of such a surfactant which we have found to be useful is that available under the name Adogen 464.

A further surfactant which may be used is Aliquat 336.

A preferred non-ionic surfactant is a polyoxyethylene sorbitan monooleate having for example 15 to 25 oxyethylene units in the chain. e.g. polyoxyethylene 20 sorbitan monooleate. A suitable example of such a surfactant is available under the name Tween-80.

We have found that certain polymer/surfactant combinations are to be preferred as membranes for particular types of sensor. Thus, in the case of pyruvate and lactate sensors of the type given by (i) and (ii) in Table 1, it is preferred that the membrane is cellulose acetate modified by a methyl trialkyl quaternary ammonium chloride (e.g. Adogen 464), most preferably such that the surfactant provides about 50% by weight of the total weight of the membrane. However in the case of a malate sensor as represented by (iii) in Table 1, it is preferred that the membrane comprises cellulose acetate modified with a polyoxyethylene sorbitan monooleate (e.g. Tween 80), most preferably such that the surfactant provides about 50% by weight of the total weight of the membrane. This leads to a further aspect of the present invention in that we have ascertained that a membrane incorporating a cationic surfactant has the ability to select between similar sized organic molecules such as malate and pyruvate, possibly on the basis of the extent of ionisation at a given pH (malic acid being dibasic and pyruvic acid being monobasic).

Therefore according to a second aspect of the present invention there is provided a biosensor for the determination of a selected analyte in a sample, the bio-

sensor comprising an enzyme layer incorporating an enzyme which is capable of interacting with said analyte to provide a detectable change, detecting means on one side of said layer for detecting said change, and an outer diffusion limiting barrier membrane which is provided on the opposite side of said layer to the detecting means, said membrane incorporating a surfactant so as to render it permeable to said analyte wherein said surfactant is a cationic surfactant.

The enzyme layer of the sensor of the second aspect of the invention preferably (but not necessarily) incorporates substances essential for the activity of the enzyme, i.e. as described for the first aspect of the invention. If such substances are not incorporated in the enzyme layer they may be provided in the sample to be analysed.

The preferred surfactant for use in the sensor of the second aspect of the invention is a methyl trialkyl quaternary ammonium salt of the type discussed above.

Therefore according to a third aspect of the present invention there is provided a membrane for use in a biosensor, said membrane incorporating a methyl trialkyl quaternary ammonium salt.

The preferred base material for the membrane of any aspect of the invention is a cellulosic material, e.g. cellulose, cellulose nitrate or a cellulose ester such as cellulose acetate or cellulose butyrate. The preferred material is cellulose acetate, preferably having an acetyl content of about 40%. The membrane preferably contains 10% to 60% by weight of the surfactant based on the total weight of the membrane.

Membranes for any aspect of the present invention may be produced by conventional casting techniques in which a solution (in a volatile solvent) of the base material of the membrane (e.g. cellulose acetate) and the requisite amount of surfactant is cast onto a flat surface and the solvent evaporated. Alternatively it is

possible to employ a "spin casting" technique in which the membrane is produced by applying a solution (of the type defined in the previous sentence) to a flat surface which is then rotated (usually about a vertical axis) at a speed which causes the solution to be evenly distributed and the solvent to be evaporated so as to produce a membrane of uniform thickness.

Typically the membrane for any aspect of the invention will have a thickness of 0.1 to 200 microns, preferably 4 to 50 microns.

The enzyme layer may be produced by immobilisation of the enzyme (and any substances necessary for the activity thereof) using conventional techniques, e.g. by incorporation in a cross-linked glutaraldehyde matrix.

If desired the enzyme layer may be laminated to at least one highly permeable support layer, e.g. a dialysis membrane.

Furthermore, the sensor of the invention may incorporate an inner membrane between the enzyme layer and the detection means for selectively preventing the passage to the detector of interferant species.

The detecting means may be an electrochemical means, most preferably of the non-potentiometric type. An amperometric detection is preferred.

The invention will be further illustrated by the following non-limiting Examples and the accompanying drawings which illustrate the results of the Example.

As described membranes were prepared from 1ml of a 5% w/v cellulose acetate in acetone containing varying amounts (%v/v) Adogen 464. The following "Conversion Table" gives the amount by weight of the surfactant in the final membrane (based on the total weight of the membrane).



Casting Solution Composition	% Adogen 464 by weight in membrane
(a) 5% Cellulose Acetate/5% Adogen	50%
(b) 5% Cellulose Acetate/4% Adogen	44%
(c) 5% Cellulose Acetate/3% Adogen	38%
(d) 5% Cellulose Acetate/2% Adogen	29%
(e) 5% Cellulose Acetate/1% Adogen	17%

All subsequent references to the Adogen (also referred to as MTAC) concentrations are to the composition of the casting solution. Thus for example reference in Fig. 1 to 5% CA/5% MTAC is to a membrane corresponding to (a) above.

#### Example 1

#### Outer Membrane for Pyruvate Biosensor

### **EXPERIMENTAL**

#### *Chemicals*

Pyruvate oxidase (EC 1.2.3.3) from *Pediococcus* species (75% protein, 80 U.mg<sup>-1</sup> protein), albumin (Bovine. Fraction V powder. 98-99% albumin), pyruvic acid (sodium salt, 99+%), cocarboxylase (thiamine pyrophosphate chloride, 98%), flavin adenine dinucleotide (FAD) (>94%), were obtained from Sigma (Poole, UK). Hydrochloric acid, cellulose acetate (39.8% acetyl content), acetone (99.9+%, HPLC grade) and Adogen 464 were from Aldrich (Poole, UK). Sodium dihydrogen-phosphate, disodium hydrogen-phosphate, magnesium chloride, sodium hydroxide, glutaraldehyde (25% solution, EM grade), aluminium oxide were from BDH (Poole, UK).

*Buffer*

A buffer comprising 18.4 mmol  $l^{-1}$   $NaH_2PO_4 \cdot H_2O$ , 81.6 mmol  $l^{-1}$   $MgCl_2$  was prepared in distilled water and adjusted to pH 7.0 with HCl or NaOH. All solutions were made up in this buffer.

*Membranes**Spin Cast Outer Membrane*

The outer membrane was formed by spin-coating 1ml of 5% w/v cellulose acetate in acetone solution containing 1-5% v/v Adogen 464 onto a 1  $cm^2$  piece of Cuprophan dialysis membrane (Gambro, Lund, Sweden) at 1000 rpm for 60s using a photo-resist spinner (E/C101D-R485, Headway Research Inc., Garland, Texas, USA).

*Enzyme Laminate Fabrication*

A composite solution of pyruvate oxidase (POD) (200 U  $ml^{-1}$ ), cocarboxylase (5 mmol  $l^{-1}$ ), Fad (5 mmol  $l^{-1}$ ),  $MgCl_2$  (1 mmol  $l^{-1}$ ) and albumin (0.1 g  $ml^{-1}$ ) was prepared in buffer solution. 10  $\mu$ L of POD-albumin solution and 5  $\mu$ l of glutaraldehyde (5% v/v in buffer) were mixed rapidly and placed on a 1  $cm^2$  portion of dialysis membrane. A further 1  $cm^2$  portion of dialysis membrane was then placed on top, and glass plates were used to compress the enzyme film so that it was evenly distributed between the membranes. The crosslinked enzyme / membrane laminate was allowed to air-dry for 10 min then washed in buffer to remove excess glutaraldehyde. The laminate was used in all experiments, with the additional modified cellulose acetate-coated membrane placed on the upperside.

*Apparatus and Electrode Assembly*

The amperometric cell (Rank Brothers, Bottisham, UK) consisted of a central 2 mm diameter platinum working electrode with an outer concentric 12 mm diameter and 1 mm wide silver ring (Ag/Ag Cl) as a counter / reference electrode. Before use, electrodes were polished with wet and then dry aluminium oxide powder. The electrodes were then covered with a small volume of buffer and the enzyme laminate

plus the additional outer membrane was placed over the electrodes. The working electrode was polarised at +650 mV (vs. Ag/AgCl) for the oxidation of enzymatically generated H<sub>2</sub>O<sub>2</sub>, using a potentiostat (Chemistry Workshops, University of Newcastle, UK) with an output to a chart recorder (Lloyd Instruments, Fareham, UK) for recording of the current/time response.

#### *Pyruvate Response*

Baseline current in buffer (<5 nA) was attained before measurement. 1 ml of 5 mmoles l<sup>-1</sup> of buffered pyruvate solution was added to the sample chamber and the current/time response was monitored. Between successive exposures the sample chamber was rinsed three times with buffer and left to recondition for 30 min.

#### Results

Fig. 1 shows the ability of the CA/Adogen (MTAC) membrane to retain the essential cofactors (cocarboxylase, FAD, Mg<sup>2+</sup>) allowing a reagentless, reusable pyruvate sensor. With a dialysis outer membrane only, the sensor rapidly loses activity as the cofactors are lost. Fig. 2 shows a similar effect for a range of Adogen concentrations and contrasts with unmodified cellulose acetate where cofactors are retained but the membrane is very impermeable to pyruvate.

#### Example 2

##### Outer Membrane of Lactate Sensor

The most effective membrane for a reagentless lactate sensor was found to be 5%CA/5% Adogen. The method of fabricating the membrane/sensor was the same as for pyruvate except:

##### *Enzyme Laminate Fabrication*

4µl of lactate dehydrogenase (LDH) (1000 U ml<sup>-1</sup>) in buffer was mixed with 11µl of diaphorase (181.5U ml<sup>-1</sup>) in buffer containing 0.75 mmoles l<sup>-1</sup> potassium

ferricyanide and  $0.75 \text{ mmoles l}^{-1} \text{ NAD}^+$ .  $15 \mu\text{L}$  of the composite solution were dropped onto a  $1 \text{ cm}^2$  piece of  $0.45 \mu\text{m}$  mixed cellulose ester HA membrane (Millipore) and air-dried for 30 min. A further  $1 \text{ cm}^2$  portion of modified cellulose acetate-coated dialysis membrane was then placed on top (see pyruvate), and the laminate was positioned on the amperometric cell as above.

### Example 3

#### Outer Membrane for Malate Sensor

The optimum membrane for a reagentless malate sensor was found to be 5%CA/5% Tween-80.

Methods as above, except:

#### *Enzyme Laminate Fabrication*

$15 \mu\text{l}$  of a composite solution of malate dehydrogenase (MDH) ( $250 \text{ U ml}^{-1}$ ), diaphorase ( $125 \text{ ml}^{-1}$ ), potassium ferricyanide ( $50 \text{ U ml}^{-1}$ ) and  $\text{NAD}^+$  ( $50 \text{ U ml}^{-1}$ ) were dropped into a  $1 \text{ cm}^2$  piece of  $0.45 \mu\text{m}$  mixed cellulose ester HA membrane (Millipore) and air-dried for 30 min. A further  $1 \text{ cm}^2$  portion of modified cellulose acetate-coated dialysis membrane was then placed on top (see pyruvate), and the laminate was positioned on the amperometric cell as above.

### **Results**

Fig. 3 shows how NAD and ferricyanide are rapidly lost from a sensor when a dialysis membrane is used as an outer membrane. Note that the sensor is regenerated by the addition of NAD/ferricyanide in solution. Fig. 4 shows the effect of varying Tween content. If the Tween content is too high the membrane is too permeable and the cofactor/mediator are lost, and if the Tween content is too low the membrane is impermeable to malate.

CLAIMS

1. A biosensor for the determination of a selected analyte in a sample, the biosensor comprising an enzyme layer incorporating an enzyme which is capable of interacting with said analyte to provide a detectable change, detecting means on one side of said layer for detecting said change, and an outer diffusion limiting barrier membrane which is provided on the opposite side of said layer to the detecting means, said membrane incorporating a surfactant so as to render it permeable to said analyte wherein said enzyme layer incorporates at least one substance essential to enzyme activity and the nature and amount of said surfactant is such that the membrane inhibits release of said substance into the sample whilst retaining permeability to the analyte.
2. A sensor as claimed in claim 1 wherein the membrane comprises 10% to 60% by weight of surfactant based on the total weight of the membrane.
3. A sensor as claimed in claim 1 or 2 wherein the surfactant is a cationic surfactant.
4. A sensor as claimed in claim 3 wherein the cationic surfactant is a methyl mixed trialkyl quaternary ammonium salt in which said alkyl groups have from 10 to 100 carbon atoms.
5. A sensor as claimed in claim 1 or 2 wherein the surfactant is a non-ionic surfactant.
6. A sensor as claimed in claim 5 wherein the non-ionic surfactant is a polyoxyethylene sorbitan monooleate.

7. A sensor as claimed in any one of claims 1 to 6 wherein the base material of the membrane is a cellulosic material.
8. A sensor as claimed in claim 7 wherein the base material of the membrane is cellulose, cellulose nitrate or a cellulose ester.
9. A sensor as claimed in claim 8 wherein the base material of the membrane is cellulose acetate.
10. A sensor as claimed in claim 9 wherein the cellulose acetate has an acetyl content of 35% to 40% by weight.
11. A sensor as claimed in any one of claims 1 to 10 wherein the membrane has a thickness of 0.1 to 200 microns.
12. A sensor as claimed in any one of claims 1 to 11 wherein the detecting means is an electrochemical means.
13. A sensor as claimed in claim 12 wherein the detecting means is an amperometric electrode.
14. A sensor as claimed in any one of claims 1 to 13 wherein the enzyme is pyruvate oxidase.
15. A sensor as claimed in claim 14 wherein the enzyme layer incorporates Cocarboxylase (thiamine pyrophosphate chloride),  $Mg^{2+}$ , and flavin adenine dinucleotide (FAD)
16. A sensor as claimed in any one of claims 1 to 13 wherein the enzyme is lactate dhydrogenase.

17. A sensor as claimed in claim 16 wherein the enzyme layer contains Diaphorase, potassium ferricyanide, and  $\text{NAD}^+$ .
18. A sensor as claimed in any one of claims 1 to 13 wherein the enzyme is malate dehydrogenase.
19. A sensor as claimed in claim 18 wherein the enzyme layer contains Diaphorase, potassium ferricyanide, and  $\text{NAD}^+$ .
20. A biosensor for the determination of a selected analyte in a sample, the biosensor comprising an enzyme layer incorporating an enzyme which is capable of interacting with said analyte to provide a detectable change, detecting means on one side of said layer for detecting said change, and an outer diffusion limiting barrier membrane which is provided on the opposite side of said layer to the detecting means, said membrane incorporating a surfactant so as to render it permeable to said analyte wherein said surfactant is a cationic surfactant.
21. A membrane for use in a biosensor, said membrane incorporating a methyl trialkyl quaternary ammonium salt.

1-4

Expt. 196/7. PO Dialysis Laminate. 21.6.95 PO soln. + 5mM TPP/FAD  
5mM pyruvate + 1mM MgCl<sub>2</sub>, 100mM phosphate, pH 7.0

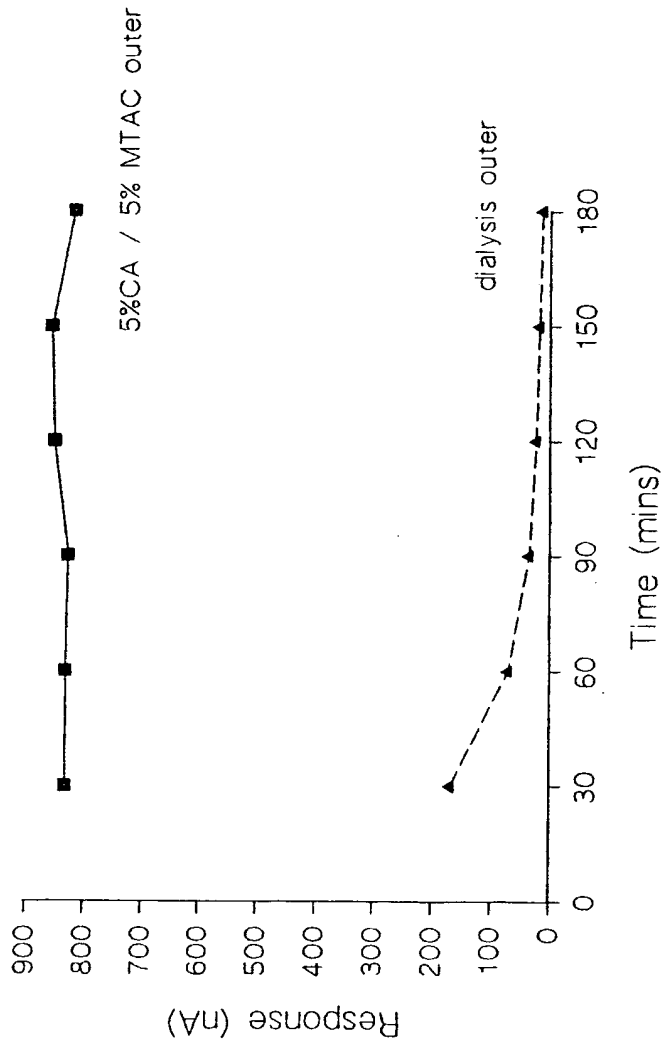


FIG. 1



2-4

Fig. 1.1. POD Dialysis Laminar + TPP/FAD  
Outer Membrane: 5% CA / x% MTAC (1000rpm, 1ml, 60s)  
5mM pyruvate + 1mM MgCl<sub>2</sub>, 100mM phosphate, pH 7.0

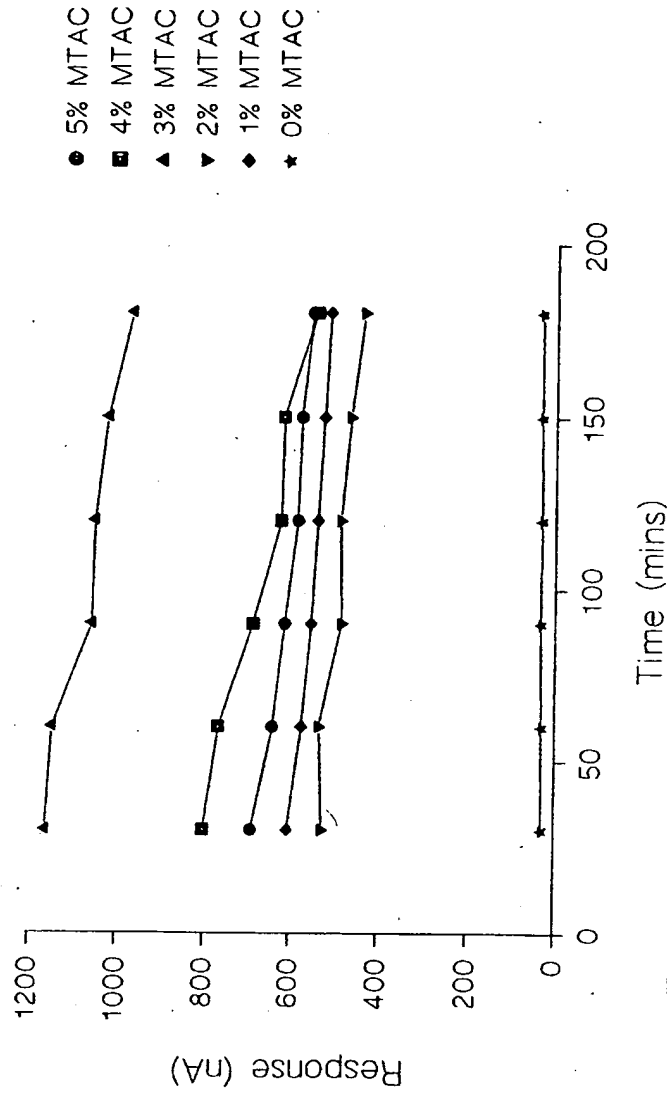
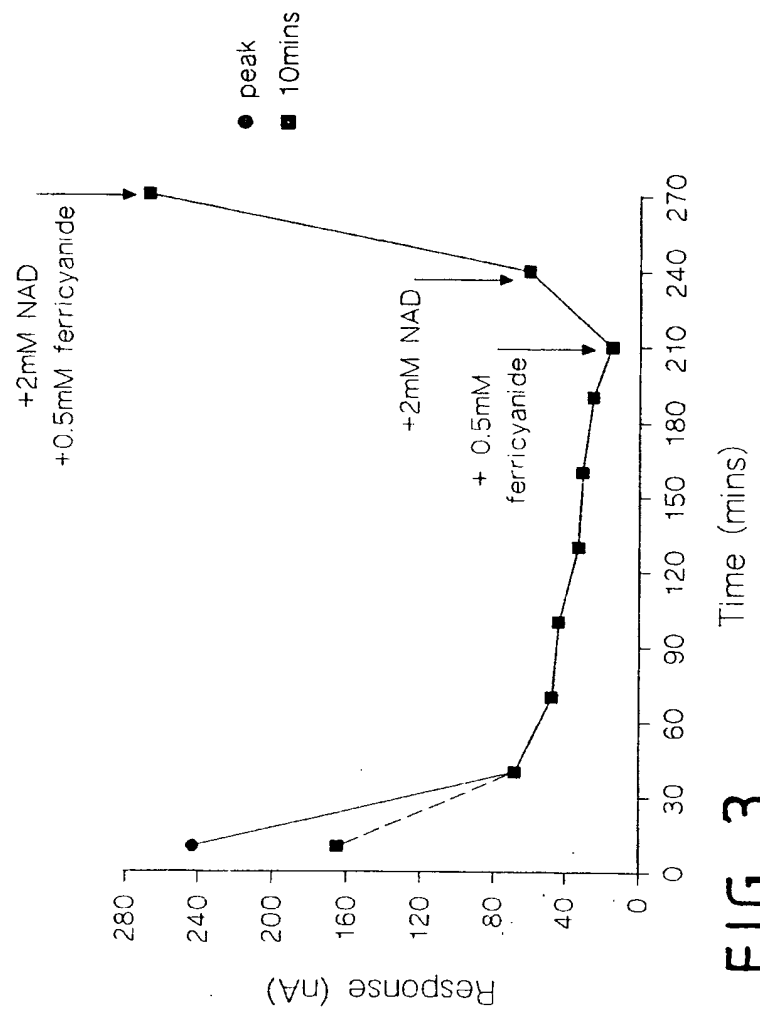


FIG. 2

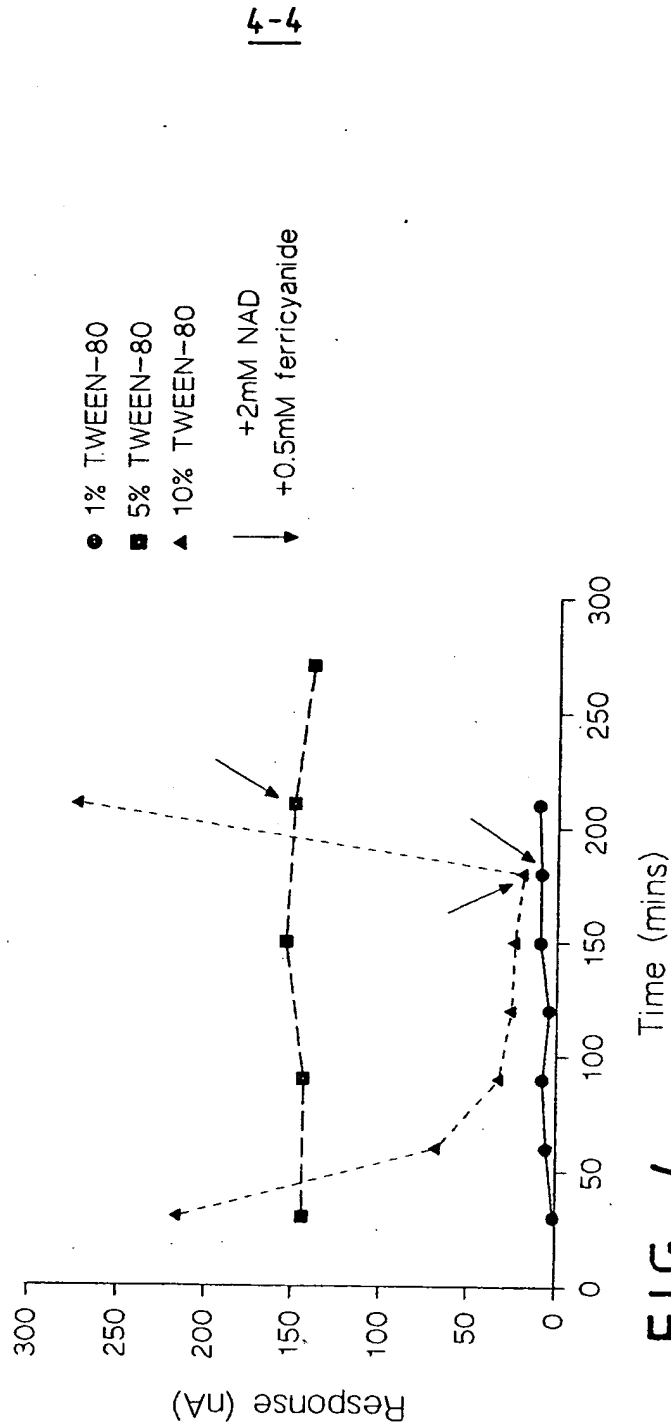
3-4

Fig. 1.7. MDH/DI/NAD/ferricyanide on HA  
Dialysis Outer. 10mM malate, pH 7.8



**FIG. 3**

Fig. 1.10. MDH/DI/NAD/ferricyanide on HA  
5%CA/x%TWEEN-80 OUTER (on Dialysis)  
10mM malate, pH 7.8



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 97/02948

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 G01N27/327 C1201/00				
According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b>				
Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12Q G01N				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
Y	EP 0 415 124 A (NIPPON ELECTRIC CO) 6 March 1991 see page 2, column 1, line 41 - column 2, line 46 ---	1-21		
Y	DATABASE WPI Section Ch, Week 8542 Derwent Publications Ltd., London, GB; Class A96, AN 85-259799 XPO02054467 & JP 60 173 457 A (MATSUSHITA ELEC IND CO LTD) . 6 September 1985 see abstract ---	1-21		
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Date of the actual completion of the international search	Date of mailing of the international search report			
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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C			

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C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PATENT ABSTRACTS OF JAPAN vol. 016, no. 272 (P-1373), 18 June 1992 & JP 04 070558 A (YAMAGUCHIKEN), 5 March 1992, see abstract ---	1-21
A	DATABASE WPI Section Ch, Week 9421 Derwent Publications Ltd., London, GB; Class A89, AN 94-170503 XP002054468 & JP 06 109 692 A (A & D KK) , 22 April 1994 see abstract ---	1,20
A	DATABASE WPI Section Ch, Week 8129 Derwent Publications Ltd., London, GB; Class A96, AN 81-52562D XP002054469 & JP 56 064 789 A (TOKYO SHIBAURA ELECTRIC CO) . 2 June 1981 see abstract ---	1,20
A	US 5 540 828 A (YACYNICH ALEXANDER) 30 July 1996 see the whole document ---	1,20
P,A	US 5 628 890 A (CARTER NIGEL F ET AL) 13 May 1997 see the whole document -----	1,20

**INTERNATIONAL SEARCH REPORT**

information on patent family members

International Application No

PCT/GB 97/02948

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0415124 A	06-03-91	JP 3065644 A	20-03-91
		JP 2508297 B	19-06-96
		JP 3122560 A	24-05-91
		DE 69023430 D	14-12-95
		DE 69023430 T	18-07-96
		US 5196340 A	23-03-93
US 5540828 A	30-07-96	US 5286364 A	15-02-94
US 5628890 A	13-05-97	CA 2159553 A	30-03-97
		JP 9222411 A	26-08-97